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Glyceraldehyde-3-phosphate activates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase

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Nitric oxide was recently demonstrated to stimulate ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Our studies on the effect of glyceraldehyde-3-phosphate (GA3P), the natural substrate of dehydrogenase activity of GAPDH, indicated GA3P to be another very potent activator of ADP-ribosylation of the enzyme. GA3P was able to activate ADP-ribosylation only in the presence of DTT. The action of GA3P was associated with inhibition of GAPDH dehydrogenase activity K_a for GA3P was at least 50-fold lower and maximal activation was somewhat higher than these values for other aldehydes that were also able to enhance GAPDH ADP-ribosylation in the presence of DTT. ADP-ribosylation was blocked by carboxamidomethylation of the essential cysteine SH-group. The bond between the prelabeled protein and ADP-ribose was resistant to hydrolysis with hydroxylamine and $HgCl_2$, suggesting that a lysine ε-amino group is the target for ADP-ribosylation.

ADP-ribosylation; Glyceraldehyde-3-phosphate; Glyceraldehyde-3-phosphate dehydrogenase

1. INTRODUCTION

NAD^+ is a cofactor of many dehydrogenases. In the mean time NAD^+ can be cleaved by ADP-ribosyl transferases so that the ADP-ribose moiety becomes attached to an amino acid acceptor residue in target proteins. ADP-ribosyl transferase activity is attributable to diverse bacterial toxins. Also intracellular or endogenous ADP-ribosylation reactions have been shown to exist in vivo [1,2]. Recently the substrate of nitric oxide-activated ADP-ribosylation has been described in many cell types [3]. The protein was purified and identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by Kots et al. [4], Dimmeler et al. [5], and Zhang and Snyder [6]. ADP-ribosylation of GAPDH may be involved in the NO-mediated cGMP-independent intercellular signalling in blood vessels, macrophage and neuron cytotoxicity [7], as well as the hepatocyte metabolism suppression [8].

In the present study we have found that ADP-ribosylation of GAPDH is activated not only by nitric oxide, but also by D-glyceraldehyde-3-phosphate (GA3P), the natural substrate for dehydrogenase activity of GAPDH, and other aldehydes.

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GA3P, glyceraldehyde-3-phosphate; DTT, dithiothreitol.

2. MATERIALS AND METHODS

2.1 *Materials*

[Adenylate-^{32}P]NAD^+ (1000 Ci/mmol) was synthesized from [α-^{32}P]ATP (Amersham, UK) as described [9]. [^{14}C]Iodoacetamide (52 mCi/mmol) was purchased from Amersham, UK. Reagents for electrophoresis were from Bio-Rad, USA. GA3P, D-glyceraldehyde and NAD^+ were from Serva, Germany. All other reagents were of the analytical grade.

GAPDH was extracted from rabbit muscle and the extract was fractionated as described [10]. The acyl group was removed from the enzyme's active site by incubation in 20 mM Tris-HCl (pH 7.4) containing 0.3 mM NAD^+ and 0.4 mM sodium arsenate at 25°C for 10 min [11]. GAPDH was further recrystallized by addition of saturated ammonium sulfate (pH 8.2) four times. GAPDH preparations (20 μg) exhibited no detectable impurities in SDS-PAGE analysis. Before the assays, the enzyme was incubated with 100-fold excess of NAD^+ in order to displace possible ADP-ribose present in the coenzyme-binding site [11] and desalted by passing through a Sephadex G-50 column equilibrated with 20 mM Tris-HCl (pH 7.5). Routinely this preparation contained 3.5–3.9 moles of NAD^+ per mole of tetramer The enzyme and nucleotide concentrations were determined spectrophotometrically from the A_{280}/A_{260} ratio [12].

2.2. *[^{32}P]ADP-ribosylation assay*

GAPDH (2 μg) was added to the incubation mixture (final volume 40 μl) containing 20 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol (DTT), 2 μM NAD^+, 1 μCi [^{32}P]NAD^+, 0.1 mM nitroprusside, or 0.5 mM GA3P, or other aldehydes as indicated. Incubation was carried out for 1 h at 37°C and the reaction was stopped by the addition of the denaturation mixture [13]. Samples were boiled for 3 min and electrophoresed through 11% SDS-PAGE by the method of Laemmli [14]. Gels were fixed, stained with Coomassie R-250, destained in boiling water and dried. Dry gels were exposed to Amersham Hyperfilm MP (preflashed) in the intensifying screen cassette for 20 h. Bands of interest were excised from the gel and counted by liquid scintillation. Relative extent of labeling was also quantified by densitometry of autoradiograms (three times each lane) with subsequent determination of peak areas using an Ultroscan XL laser densitometer (LKB,

Sweden) linked to an Olivetti M-290 personal computer driven by Gelscan XL software. The linearity of peak area versus degree of labeling was verified in a separate experiment.

2.3. *Other assays*

GAPDH was carboxamidomethylated in 20 mM sodium pyrophosphate (pH 8.5) Samples containing 10 μM enzyme and 100 μM [^{14}C]iodoacetamide were incubated for suitable time intervals (0.5–15 min) at 20°C. Modification was stopped by addition of DTT to a final concentration of 10 mM, and [^{32}P]ADP-ribosylation of GAPDH in the presence of 1 mM glyceraldehyde and 10 mM DTT was assayed.

Dehydrogenase activity of GAPDH was measured at 25°C in 50 mM potassium phosphate, 50 mM glycine (pH 9.0), 0.5 mM NAD^+ and 0.5 mM GA3P. Reaction was started by addition of 1 μg of GAPDH and increase in absorbance at 340 nm was estimated.

All experiments were repeated three times and means ± S.E.M. ($P < 0.05$) are presented.

3. RESULTS AND DISCUSSION

Nitroprusside-activated ADP-ribosylation of GAPDH from human erythrocytes [4] and other sources [6] is known to be characterized by low stoichiometry. To find the proper explanation of this fact we have studied the effect of GA3P, the natural substrate for dehydrogenase activity of GAPDH, on its ADP-ribosylation. Nitroprusside did greatly stimulate (approx. 20-fold) ADP-ribosylation of the rabbit muscle enzyme (Fig. 1). When the protein was incubated in the presence of both nitroprusside and GA3P, the labeling of the 37 kDa polypeptide was reduced 6-fold. The inhibition of the nitroprusside action may be the result of rapid inter-reaction of nitroprusside with the aldehyde, thus preventing NO liberation and, at the same time, inactivating GA3P [15]. The effect of NO on ADP-ribosylation was unexpectedly mimicked by GA3P. The label incorporation dramatically increased approx. 25-fold in samples containing both GA3P and DTT relative to the control samples containing DTT alone (Fig. 1). Thus, apart from nitric oxide, ADP-ribosylation of GAPDH is also activated by GA3P in a manner similar

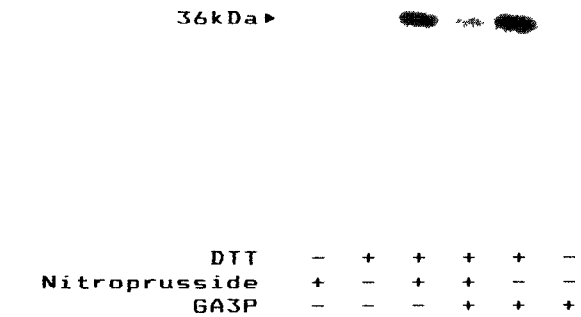

Fig. 1. Effects of nitroprusside, GA3P, and DTT on ADP-ribosylation of GAPDH. SNP, sodium nitroprusside. ADP-ribosylation was assayed as described in section 2.2, and the autoradiogram shown is representative of four experiments.

to the nitroprusside-dependent modification, as GA3P exerts its effect only in the presence of DTT. Moreover, one can suggest that in this case auto-modification of the enzyme occurs, because the GAPDH preparation used showed no detectable impurities.

The effect of GA3P was time-dependent (Fig. 2A). Following a 20 min lag-period, rapid increase in ADP-ribose incorporation was observed and a stable plateau was reached after 1.5 h incubation. Dehydrogenase activity of GAPDH was tested in parallel (Fig. 2B) and found to be inhibited under the same conditions used for ADP-ribosylation assay. It is noteworthy that the inhibition had approx. the same lag-period (about 20 min) followed by rapid inactivation up to 70% of control samples' activity after 1 h of incubation. This fact suggests that GAPDH may be inhibited due to ADP-ribosylation, but that is not apparently true because only 4 ± 1% of GAPDH tetramers were ADP-ribosylated, while dehydrogenase activity was inhibited by

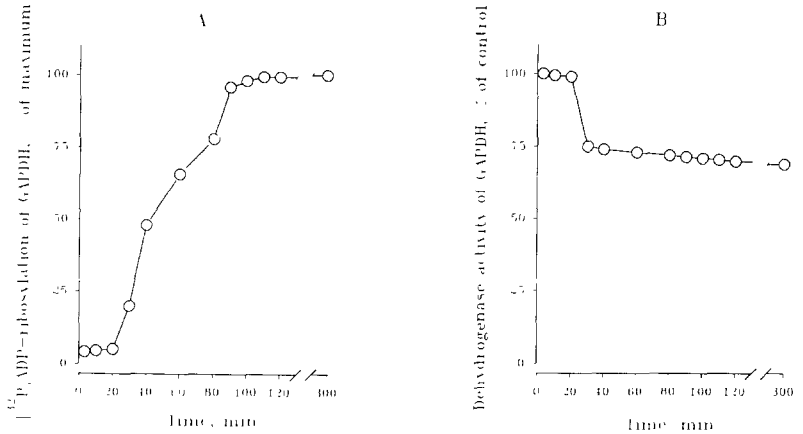

Fig. 2. Time-dependent activation of GAPDH ADP-ribosylation and inhibition of enzyme dehydrogenase activity by GA3P. GAPDH was incubated in the presence of GA3P and DTT as described in section 2.2. At indicated time intervals aliquots were either analyzed by SDS-PAGE and [^{32}P]ADP-ribosylation was estimated by laser densitometry of autoradiograms (A), or assayed for dehydrogenase activity (B). Protein treated with 10 mM DTT served as the control. The data shown are representative of three experiments.

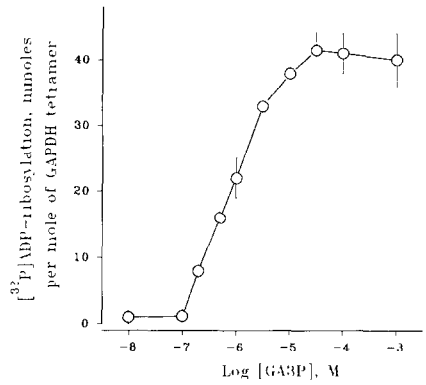

Fig. 3. Effect of GA3P concentration on GAPDH ADP-ribosylation Protein was incubated in the presence of 10 mM DTT and GA3P concentrations as indicated. Label incorporation was counted by liquid scintillation.

28 ± 3%. More likely, ADP-ribosylation is the result of change in the conformation of either all or part of enzyme molecules in the presence of GA3P and DTT.

The effect of GA3P on GAPDH ADP-ribosylation was concentration-dependent (Fig. 3). K_a value estimated was 2.7 ± 1.9 μM (Table I). This is almost 20-fold lower than the K_m value for dehydrogenase activity of GAPDH [10]. Thus ADP-ribosylation is probably unrelated to oxidation of GA3P, as well as to phosphorolysis of the acylated enzyme, because phosphate and arsenate in concentrations up to 20 mM had no effect on GA3P-activated ADP-ribosylation (data not shown).

To assess the specificity of GA3P effect relative to other compounds, the ability of several aldehydes listed in Table I to promote ADP-ribosylation of GAPDH was tested. All substances were able to activate modification in the DTT-dependent manner. These aldehydes had K_a values at least 50-fold higher than that of GA3P, and their maximal effect on ADP-ribosylation was at least 1.5-fold less. Thus we conclude that GA3P is a specific stimulator of GAPDH ADP-ribosylation.

Table I

Activation of GAPDH ADP-ribosylation by aldehydes

Substance	K_a (μM)	Maximal activation (% of control)
GA3P	2.7 ± 1.9	2340 ± 275
Glyceraldehyde	120 ± 10	1692 ± 106
Benzaldehyde	305 ± 110	229 ± 139
Salicylic aldehyde	1750 ± 300	298 ± 139
Glycolic aldehyde	2200 ± 900	714 ± 61

GAPDH was ADP-ribosylated in the presence of 10 nM - 25 mM of indicated compound, label incorporation was measured by densitometry of autoradiograms, and K_a value was calculated. Maximal activation was estimated in the presence of 5 mM of indicated compound, and radioactivity was counted by liquid scintillation.

As shown in Fig. 4, aldehyde-induced ADP-ribosylation was strongly inhibited by carboxamidomethylation of GAPDH. The essential Cys^{149} SH-group in the active site of enzyme is known to react with iodoacetamide [16]. We suppose that this SH-group is involved in ADP-ribosylation. Furthermore, the non-linear concave shape of the inhibition curve suggests that the tetramer subunits interaction plays a certain role in ADP-ribosylation. The stimulatory effect of GA3P was not observed on the dimeric enzyme from human erythrocytes (data not shown) used instead of the rabbit muscle tetramer, so the intact quaternary structure of GAPDH is evidently necessary for ADP-ribosylation.

Nitroprusside was shown to activate cysteine-specific ADP-ribosylation of GAPDH [4,6,20]. In contrast to this, in the case of GA3P- as well as glyceraldehyde-stimulated ADP-ribosylation the bond between the protein and ADP-ribose was not significantly hydrolyzed by the treatment of the prelabeled protein with $HgCl_2$ or hydroxylamine, but was cleaved by phosphodiesterase (Table II). This fact indicates that the modified amino acid residue is neither arginine, asparagine, cysteine nor a carboxyl group, because these amino acids form bonds with ADP-ribose known to be cleaved by NH_2OH or $HgCl_2$ [17]. An alternative may be a lysine residue ε-amino group, whose conjugates with ADP-ribose are resistant to hydrolysis conditions indicated in Table II [18]. The possibility that a lysine residue is ADP-ribosylated in GAPDH has been mentioned by other authors [19]. In general our results indicate that nitric oxide is not unique in its ability to promote ADP-ribosylation of GAPDH. Although aldehydes induce apparently lysine-specific ADP-ribosylation, while NO activates cysteine-specific modification, the strong similarity between these modifications exists in requirement for DTT and for the intact essential SH-group ([20] and Fig. 4). Nevertheless it is possible to propose that alde-

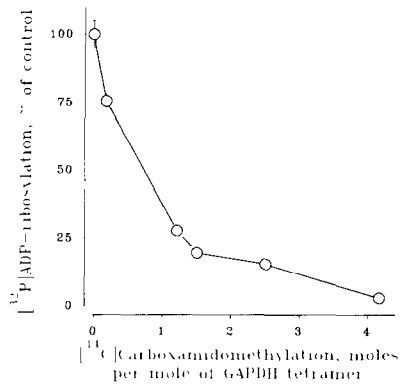

Fig. 4. Inhibition of glyceraldehyde-stimulated ADP-ribosylation by carboxamidomethylation. GAPDH was incubated with [^{14}C]iodoacetamide and then [^{32}P]ADP-ribosylated as described in section 2.3. Gel was dissolved in 30% H_2O_2, 1% NH_4OH and dual label was counted by liquid scintillation Correlation coefficient between ^{14}C- and ^{32}P-label incorporation was −0.98 ± 0.28. ($P < 0.05$).

Table II

Stability of the bond between GAPDH and ADP-ribose

Activator used for stimulating ADP-ribosylation	Radioactivity remaining bound to GAPDH after indicated treatment of prelabeled protein (% of control)		
	Hydroxylamine	$HgCl_2$	Phosphodiesterase
Nitroprusside, 0.1 mM	94 ± 14	5 ± 12	< 2
GA3P, 0.5 mM	91 ± 8	68 ± 7	< 2
Glyceraldehyde, 1 mM	88 ± 11	79 ± 19	< 2

ADP-ribosylation assays were stopped by addition of 5% trichloroacetic acid, 0.1% bovine serum albumin. Protein was pelleted and resuspended in 0.1 M HEPES-NaOH (pH 7.5) and treated with 1 M hydroxylamine (pH 7.0) for 3 h, 1 mM or 10 mM $HgCl_2$ for 30 min, or with 50 μg/ml snake venom phosphodiesterase, 10 mM $MgCl_2$ for 30 min at 37°C. Protein was repelleted, samples were electrophoresed, and label incorporation was measured by densitometry of autoradiograms.

hydes may non-selectively react with many amino acid residues and hence cause NAD^+ cleavage with the subsequent ADP-ribose transfer.

The functional significance of GAPDH auto ADP-ribosylation is presently unknown. This modification may affect not only dehydrogenase activity of the enzyme but also other functions of GAPDH including the triad junction formation [21], tRNA translocation catalysis [22], and uridine DNA glycosylase activity [23]. Thus GAPDH being the multifunctional protein may be regulated via its ADP-ribosylation by intracellular concentrations of NO, GA3P, other aldehydes and reduced thiol-containing agents.

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